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Rapid Communication

Cytokine-activated Human Endothelial Cells Synthesize and Secrete a Monocyte Chemoattractant, MCP-1/JE

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We have demonstrated inducible expression of the mRNA encoding the monocyte chemoattractant MCP-1, the human homolog of the JE gene, in endothelial cells within 3 hours of treatment with IL-1β and tumor necrosis factor. IFN-γ also induced expression of this mRNA after 24 hours, but to a lesser extent. MCP-1/JE protein steadily accumulated in the medium of endothelial cells during a 48-hour exposure to IL-1β. Medium conditioned by IL-1β–treated endothelial cells contained monocyte chemoattractant activity that was immunoadsorbed by anti–MCP-1 antibodies. These results suggest that endothelial cells secrete a monocyte chemoattractant, MCP-1/JE, in response to inflammatory mediators, and thus may contribute to the accumulation of monocytes at sites of inflammation. (Am J Pathol 136:1229–1233)

Human vascular endothelial cells participate in acute inflammatory and immune reactions and are believed to play a role in chronic vascular abnormalities, such as atherosclerosis.[1,2] Local infiltration by various leukocyte populations is a prominent characteristic of these conditions. Activated vascular endothelium can contribute to inflammation by stimulating leukocyte migration. For example, cultured endothelial cells treated with IL-1[1] or TNF secrete the neutrophil chemoattractant NAP-1 (also called IL-8, MDNCF, NAF, or 3-10C).[3] A structurally related monocyte chemoattractant, called MCP-1,[4,5] MCAF,[6,7] or SMC-CF,[8,9] has also been described. It has been identified in culture fluids of many mammalian cell types, including tumor cells,[4] smooth muscle cells,[9] and phytohemagglutinin-stimulated blood mononuclear cells.[10] The gene encoding this protein is the human homolog of the platelet-derived growth factor (PDGF)[1]-inducible murine gene *JE*.[11] Recently it has been shown that IL-1β and TNF also induce the expression of MCP-1/JE mRNA in endothelial cells,[12] and that minimally modified LDL induces the secretion of SMC-CF.[13] We show here that human endothelial cells express MCP-1/*JE* mRNA after treatment with IL-1β, TNF, and IFN-γ, that endothelial cells secrete an immunoreactive form of MCP-1/JE protein, and that this protein accounts for most of the monocyte chemoattractant activity produced by endothelial cells in response to IL-1β.

Materials and Methods

Cell Culture and Cytokines

Human endothelial cells were isolated from 3 to 5 umbilical veins by collagenase digestion, pooled, and serially subcultured. Cells were maintained in medium containing fetal calf serum and endothelial cell growth factor. Culture conditions and reagents have been described elsewhere.[14] Cytokines used in these studies included recombinant human IL-1β (expressed in *Escherichia coli,* 1.0 × 10^7 U/mg, a gift of Dr. A. Shaw, Glaxo, Geneva, Switzerland), recombinant human tumor necrosis factor (TNF)

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(expressed in *E. coli,* 2.5×10^7 U/mg, a gift of Biogen, Cambridge, MA), and recombinant IFN-γ (expressed in Chinese hamster ovary cells, 2.5×10^5 U/ml conditioned medium, a gift of Professor W. Fiers, State University of Ghent, Belgium). All of the cytokine preparations were active in appropriate bioassays and were completely neutralized by relevant specific antisera. All experiments were conducted at subcultures 3 to 5, by which time mononuclear phagocytes were not detectable in these cultures.

RNA Analysis

RNA was isolated from 3 to 5×10^6 cells by guanidinium isothiocyanate extraction followed by centrifugation through cesium chloride.[15] RNA was electrophoretically fractionated through a 1.5% agarose 12.2 mol/l (molar) formaldehyde gel and transferred to nitrocellulose filters. Baked filters were hybridized at 42°C as described.[16] Probes were nick translated to a specific activity of more than 10^8 CPM/μg, and included *JE,* the XhoI fragment from phJE-34[11]; γIP-10, the PstI fragment from plFNγ-31,[17] a gift of Dr. A. Luster (Massachusetts General Hospital, Boston, MA); and NAP-1, a PstI-EcoRI fragment from the IL-8 cDNA cloned into pKK233-2, a gift of Dr. S. L. Kunkel (University of Michigan Medical School, Ann Arbor, MI). Densitometry measurements were performed using an LKB Ultrascan enhanced laser densitometer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Immune Precipitation

Two T75 flasks (Corning, Corning, NY) each containing 2×10^6 confluent endothelial cells were treated with 250 μCi/ml [^{35}S]-methionine (DuPont NEN, Boston, MA) in complete medium for 30 minutes, after which 10 U/ml IL-1β was added to one flask. At the indicated times, 0.5 ml medium was withdrawn from each flask and made 2 mmol/l (millimolar) in PMSF. Cells and debris were removed by centrifugation, an equal volume of RIPA buffer (50 mmol/l TRIS-HCl [pH7.5], 150 mmol/l NaCl, 0.1% SDS, 1% Triton X-100, 0.5% deoxycholate, 1 mmol/l PMSF) was added, and immune precipitation carried out with anti-JE antiserum or control serum as described.[11]

Preparation of Endothelial Cell Conditioned Medium

A total of 50×10^6 endothelial cells were treated for 24 hours with 10 U/ml IL-1β. After ultrafiltration of a portion of the medium using a YM-5 filter (Amicon, Danvers, MA), it was fractionated by HPLC gel filtration using a 7.5×600 mm TSK-2000 column (Toya Soda, Tokyo, Japan) as described.[18]

Immunoadsorption of Monocyte Chemotactic Activity

Twenty-five microliters (packed volume) of Protein A-Sepharose was rinsed with Gey's balanced salt solution (GBSS) with 0.2% BSA. Fifty micrograms anti–MCP-1 IgG[18] was added to the column, which was then rinsed with GBSS/0.2% BSA. Thirty-five microliters of conditioned medium was applied to the column and the unbound fraction was eluted with 70 μl GBSS/0.2% BSA, yielding a total of 105 μl unbound material. Control columns included normal rabbit IgG Protein A-Sepharose and Protein A-Sepharose alone.[19] Monocyte chemotaxis dose-response curves were generated for each of the effluent preparations; the concentration of monocyte chemotactic activity in each was defined as the reciprocal of the dilution showing half-maximal activity.[4,18]

Monocyte Chemotaxis

Chemotaxis assays were performed in a 48-well microchamber apparatus, as described, using freshly isolated human peripheral blood mononuclear cells.[20]

Results

MCP-1/JE mRNA Expression in Endothelial Cells

Figure 1 shows that endothelial cells expressed detectable amounts of MCP-1/*JE* mRNA 3 hours after feeding them fresh medium, and that this expression decreased to nearly undetectable levels after 24 hours. After 3 hours of treatment, IL-1β and TNF induced sixfold greater increases in MCP-1/*JE* mRNA. The relative induction of MCP-1/*JE* by these cytokines was even greater after 24 hours of treatment (10- to 15-fold), although the absolute levels of expression appeared to be no higher than they were at 3 hours. IFN-γ inconsistently induced MCP-1/*JE* mRNA expression at 3 hours, but as shown in Figure 1, reproducibly induced small increases at 24 hours.

MCP-1/JE is closely related to a family of proteins that are involved in the inflammatory response,[21] including in humans the IFN-γ–inducible gene γIP-10,[17] and the neutrophil-specific chemoattractant NAP-1.[22–25] Because the genes encoding these proteins are also expressed in cytokine-activated endothelial cells, we asked whether the regulation of MCP-1/*JE* expression was similar to that of

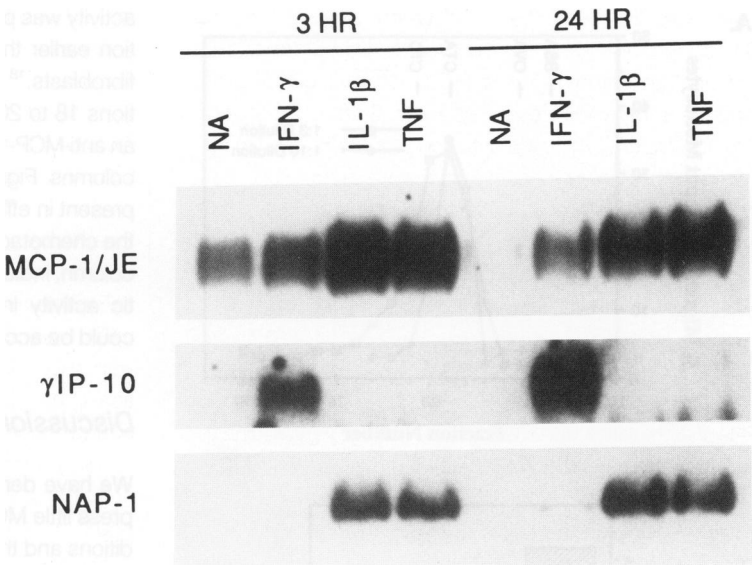

Figure 1. *Human endothelial cells were treated as indicated and their RNA was isolated and blotted onto nitrocellulose, as described in Materials and Methods. For this figure, a single blot was examined using each indicated probe, stripping the blot between experiments. (Residual MCP-1/JE probe can be seen in the γIP-10 experiment at a molecular size smaller than that of γIP-10 mRNA.) Three independent experiments demonstrated similar results. NA, no addition.*

γIP-10 or NAP-1. Figure 1 shows that of the cytokines tested, only IFN-γ induced the expression of γIP-10, while IL-1β and TNF induced NAP-1 expression. Unlike MCP-1/JE, however, NAP-1 expression was not significantly induced after 24 hours of treatment with IFN-γ.

Secretion of MCP-1/JE Protein by Endothelial Cells

As predicted by the results shown in Figure 1, cells incubated in complete medium secreted detectable amounts of MCP-1/JE protein that steadily accumulated over 48 hours (Figure 2). However, in the presence of IL-1β, cells secreted MCP-1/JE earlier (detectable at 2 hours) and to higher levels than untreated cells. The maximal difference in MCP-1/JE secretion between treated and untreated cells occurred between 10 and 24 hours after IL-1β treatment. Although the antiserum precipitates a large number of proteins, MCP-1/JE is the only protein specifically precipitated. We have further documented the presence of MCP-1/JE protein in IL-1β–treated endothelial cell conditioned medium by Western blotting. In addition, we have confirmed the specificity of this antiserum by blocking its recognition of MCP-1/JE in Western blots using excess purified recombinant MCP-1/JE (data not shown).

MCP-1/JE exists in two forms, MCP-1/JE-α of M_r 15,000, and MCP-1/JE-β of M_r 11,000,[4,11] recognized

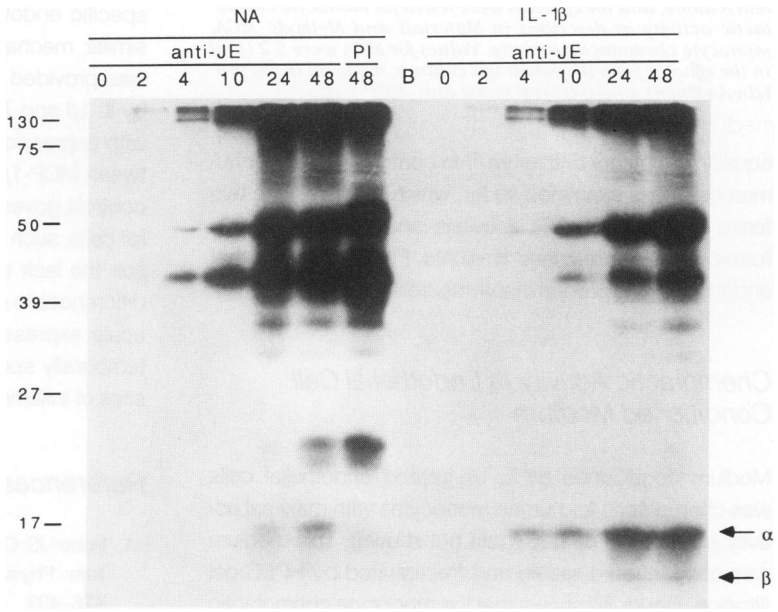

Figure 2. *Human endothelial cells were radiolabeled with [^{35}S]methionine in the absence (NA) or presence of 10 U/ml IL-1β. At the indicated times, an aliquot of medium was withdrawn and subjected to immune precipitation using anti-JE antiserum or control serum (PI). Precipitated material was analyzed by electrophoresis through an SDS-containing 17.5% polyacrylamide gel. A second experiment demonstrated similar results.*

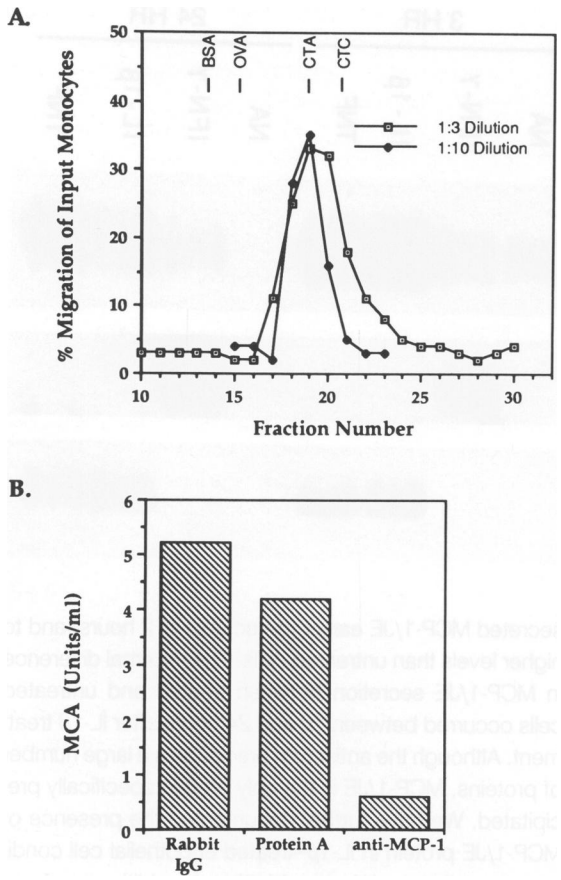

Figure 3. **A**: *Medium conditioned by IL-1β–treated human endothelial cells was concentrated and injected into a TSK-2000 HPLC gel filtration column equilibrated in PBS, and 1 ml fractions were collected at a flow rate of 1 ml/minute. Fraction aliquots were diluted in GBSS/0.2% BSA and assayed for monocyte chemoattractant activity. The column was calibrated with BSA, OVA (ovalbumin), CTA (chymotrypsinogen A), and CTC (cytochrome C).* **B**: *Medium conditioned by IL-1β–treated human endothelial cells was passed over columns containing rabbit anti–MCP-1 IgG, normal rabbit IgG, or protein A alone, and the effluents were tested for monocyte chemotactic activity as described in Materials and Methods. MCA, monocyte chemotactic activity. Values for MCA were 5.2 U/ml in the effluent from the rabbit IgG column, 4.2 U/ml in the protein A effluent, and 0.6 U/ml in the anti–MCP-1 effluent.*

equally well by our antiserum.[11] In contrast to all other human cell types examined so far, which radiolabel the two forms to equal specific activities and secrete the two forms in nearly equimolar amounts, Figure 2 shows that endothelial cells predominantly secrete the α form.

Chemotactic Activity in Endothelial Cell Conditioned Medium

Medium conditioned by IL-1β–treated endothelial cells was chemotactic for human monocytes with maximal activity at a dilution of 1:25 (data not shown). This medium was concentrated sixfold and fractionated by HPLC gel filtration. Figure 3A shows that the monocyte chemotactic activity was present as a single peak that eluted one fraction earlier than MCP-1/JE derived from glioma cells or fibroblasts.[18] To identify the chemotactic activity, fractions 18 to 20 were pooled and aliquots were applied to an anti-MCP-1 Protein A-Sepharose column and to control columns. Figure 3B shows that chemotactic activity was present in effluents from the control columns, but 88% of the chemotactic activity was removed by the anti–MCP-1 column, indicating that nearly all the monocyte chemotactic activity in the endothelial cell conditioned medium could be accounted for by MCP-1/JE.

Discussion

We have demonstrated that human endothelial cells express little MCP-1/*JE* mRNA under standard culture conditions and that a low level of expression can be induced by feeding with fresh medium. Substantially higher levels can be induced by treating cells with IL-1β and TNF, and to a lesser extent by IFN-γ. These results confirm and extend the preliminary observations of Strieter et al.,[12] and Takehara et al.[26] We report a profile of cytokine regulation that distinguishes MCP-1/JE from both NAP-1 and γIP-10. We also demonstrate that endothelial cells secrete predominantly the α form of MCP-1/JE and that this protein is the factor responsible for monocyte chemotactic activity in the medium of IL-1β–treated endothelial cells.

The secretion of a monocyte-specific chemoattractant by cytokine-activated endothelial cells provides part of the basis for the accumulation of monocytes at sites of vascular injury and inflammation. In addition to inducing chemoattraction by means of MCP-1/JE, IL-1, and TNF also cause increased adhesion of monocytes to the endothelial cell surface, probably by inducing the expression of specific endothelial–monocyte adhesion molecules.[27] A similar mechanism for the accumulation of neutrophils was provided by the demonstration of NAP-1 secretion by IL-1β and TNF-treated endothelial cells,[3] concomitant with expression of ELAM-1.[28] Despite the similarities between MCP-1/JE and NAP-1, there are differences in the controls governing their inducible expression in endothelial cells, such as the induction of MCP-1/JE by IFN-γ *versus* the lack of induction of NAP-1 by this agent. Such differences in cytokine secretion as well as adhesion molecule expression by endothelial cells may underlie the temporally specific appearance of various leukocytes at sites of inflammation *in vivo*.

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